



## Vaccination of gallinaceous poultry for H5N1 highly pathogenic avian influenza: Current questions and new technology

Erica Spackman\*, David E. Swayne

Southeast Poultry Research Laboratory, USDA-Agricultural Research Service, 934 College Station Rd., Athens, GA 30605, United States

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### ABSTRACT

Vaccination of poultry for avian influenza virus (AIV) is a complex topic as there are numerous technical, logistic and regulatory aspects which must be considered. Historically, control of high pathogenicity (HP) AIV infection in poultry has been accomplished by eradication and stamping out when outbreaks occur locally. Since the H5N1 HPAIV from Asia has spread and become enzootic, vaccination has been used on a long-term basis by some countries to control the virus, other countries have used it temporarily to aid eradication efforts, while others have not used it at all. Currently, H5N1 HPAIV is considered enzootic in China, Egypt, Viet Nam, India, Bangladesh and Indonesia. All but Bangladesh and India have instituted vaccination programs for poultry. Importantly, the specifics of these programs differ to accommodate different situations, resources, and industry structure in each country. The current vaccines most commonly used are inactivated whole virus vaccines, but vectored vaccine use is increasing. Numerous technical improvements to these platforms and novel vaccine platforms for H5N1 vaccines have been reported, but most are not ready to be implemented in the field.

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### 1. Background on H5N1 vaccination in poultry

Historically, vaccination for avian influenza virus (AIV) in poultry has not been routine for either high pathogenicity (HP) AIV or low pathogenicity (LP) AIV although it has been used in some places in recent years where AIV is present (i.e. vaccine is not used unless there is a known challenge). The H5N1 HPAIV outbreak, which began in China in 1996 and spread outside of China in Asia during 2003 then spread more widely in 2006 to Europe and Africa, has elicited the most widespread use of vaccines for HPAIV in poultry. Since 1996, 63 countries have been affected with deaths in over 250 million domestic poultry and/or wild birds (Von Dobschuetz et al., 2011). Currently, the virus is considered to be enzootic in China (including Hong Kong SAR), Bangladesh, eastern India, Indonesia, Viet Nam and Egypt (Swayne et al., 2011) (the field situation for H5N1 HPAIV is dynamic, therefore the reader is encouraged to visit the AIV portal of the world organization for animal health (OIE) for current information [[www.oie.int](http://www.oie.int)]). Of these countries, China, Indonesia, Viet Nam and Egypt vaccinate routinely. Currently (March 2013) Bangladesh is in the process of developing a vaccination policy for commercial poultry and India has no policy.

In general how vaccines have been used for controlling H5N1 HPAIV and strategies for vaccine application have varied

substantially among different countries from no vaccine use to attempts to vaccinate 100% of poultry (Swayne et al., 2011). Here we aim to discuss critical aspects of the field use of AIV vaccines for H5N1 in gallinaceous poultry, the majority of which is in chickens, and current research on poultry vaccines for H5N1. Vaccination of domestic ducks for H5N1 HPAIV is also an important topic and will be covered in a separate review (Pantin-Jackwood and Suarez, in press).

#### 1.1. Why vaccinate?

The primary goal for using vaccines has been to complement other control measures during an outbreak by reducing the amount of virus in the environment (for which there are public health implications as well) and decreasing the susceptibility of the birds to the virus. Vaccination of chickens and turkeys for AIV is nearly always to aid control of an outbreak by reducing environmental contamination and to maintain food security. However the use of vaccination to control AIV (HP or LP) and specific strategies for applying vaccine have remained controversial and numerous countries will not consider its use, while others have been quick to implement vaccination programs. There are numerous factors that affect the success of a control program for HPAIV in poultry regardless of whether vaccination is used and every outbreak is different as are the resources available to control them. Therefore there is no simple formula for whether vaccine should be used and how. It is clear that vaccine can help to break the infection cycle (Ellis et al., 2004) therefore

\* Corresponding author. Tel.: +1 706 546 3617; fax: +1 706 546 3161.

E-mail address: [Erica.spackman@ars.usda.gov](mailto:Erica.spackman@ars.usda.gov) (E. Spackman).

can aid a control program when applied properly. Approaches to control programs for AIV worldwide have been reviewed recently elsewhere (Pavade et al., 2011; Swayne et al., 2011).

### 1.2. Where is H5N1 vaccine being used?

Most vaccine for H5N1 in poultry, by dose, has been used in four countries which have ongoing vaccination programs: China (91% of vaccine doses; began vaccinating in 2004), with Indonesia (2.3%; began in 2004), Viet Nam (1.4%; began in 2005) and Egypt (4.7%; began in 2006) being among the next highest users by dose (Swayne et al., 2011). Lower quantities of vaccine for H5N1 HPAIV has been used temporarily in chickens and turkeys in several other countries: Cote d'Ivoire, France, Kazakhstan, Mongolia, Pakistan, Netherlands, Russia and Sudan (Swayne et al., 2011). Also, vaccine for control of H7 HPAI viruses has been used in North Korea (2005), Pakistan (1995) and Mexico (2012) (Swayne et al., 2011).

Specific strategies for vaccination have varied among these countries because of different resources, veterinary infrastructure, industry structures and poultry populations which are at risk. Typically commercial birds (FAO sector 1 and 2) are at lower risk for exposure due to better biosecurity; it is the sector 3 and 4 chickens (semi-commercial and village poultry, respectively) which are at the greatest risk for exposure and infection. Poultry in sector 3 and 4 operations are also frequently the most difficult populations to achieve high vaccination coverage rates. For example, vaccination coverage in sectors 3 and 4 in Egypt and Indonesia ranged from 20 to 40%, far too low for national population immunity (Swayne et al., 2011). In the commercial sector typically only long lived birds such as breeders or layers, which are more valuable and are more comprehensively vaccinated for other diseases, are targeted for AIV vaccine due to the high cost of administering the vaccine manually to each individual bird. Additionally, oil-emulsion adjuvanted vaccines require a withdrawal time prior to slaughter in most developed or transitional countries. The recommended time period for withdrawal is often longer than the life-span of meat chickens, further inhibiting vaccine use in meat poultry. However, in the current four countries where H5N1 HPAI is enzootic and that utilize vaccination, widespread H5N1 HPAIV infection has necessitated vaccination in meat poultry in addition to breeders and egg layers (Kilany et al., 2011; Swayne et al., 2011).

Importantly, the number of doses of vaccine used by a given country does not necessarily correlate with vaccine coverage and thus national flock immunity. For example the number of doses reported to be used in a country may be close to the national flock size, but some individual chickens or flocks may be vaccinated multiple times while others are not vaccinated at all. Coverage also varies substantially over time (and although not necessarily biologically pertinent, coverage is frequently estimated by year). The highest coverage, where nearly 100% of poultry are vaccinated, is in Hong Kong. Estimates of coverage vary among the other countries which have vaccinated over the course of multiple years (e.g. Indonesia, Egypt), but may be less than 20% as reported for Pakistan (Swayne et al., 2011). To achieve population immunity and prevent transmission of the virus in the population at risk, a vaccine coverage rate of minimum of 60% and optimally 80% is needed (Bouma et al., 2009; Kapczynski and Swayne, 2009).

### 1.3. What vaccines are being used?

Initially, only a few H5 inactivated oil-emulsion adjuvanted vaccines based on a limited number of seed strains were available (Table 1): A/chicken/Hidalgo(Mexico)/1994 H5N2 ("classical" lineage) (Mex/94), A/turkey/England/N28/1973 H5N3 ("classical" lineage) (England/73), A/turkey/Wisconsin/68 (H5N9) ("classical" lineage), and A/chicken/Italy/22A/1998 (H5N9) ("classical"

lineage) (Swayne and Kapczynski, 2008b). In addition, two HPAI field viruses, A/chicken/Legok(Indonesia)/2003(H5N1)(clade 2.1.1), and A/duck/Novosibirsk/02/2005(H5N1) (clade 2.2) were rapidly developed and utilized in inactivated vaccines. In 2006, Re-1, a reverse genetic engineered reassortant (rg) with the H5 hemagglutinin (HA) and N1 neuraminidase (NA) from A/goose/Guandong/1996 (clade 0) and the remaining genes from A/PuertoRico/8/1934 (PR8) was developed and used (Chen, 2009). Of these 7 vaccine seed strains, Mex/94 and Re-1 have been by far the most widely used for the H5N1 HPAIV between 2002 when the Hong Kong vaccination program was initiated through mid-2008, at the time of re-emergence of this lineage outside of China (Chen, 2009). Importantly, Mex/94 has also been used widely since 1995 in Mexico to control the H5N2 low pathogenicity (LP) AIV in chickens (Villarreal, 2009). As of the summer of 2012, Mex/94 is still used to vaccinate chickens for H5N1 HPAIV in Hong Kong, Egypt and Indonesia, but the number of doses of Mex/94 that have been used has been reduced compared to newer rg seed strains.

Re-1 was produced until early-2008 as the primary vaccine seed strain used in China with some exports of vaccine to Indonesia, Vietnam and Egypt, but Re-1 has been replaced twice with more efficacious seed strains as the field viruses have evolved antigenically and genetically (Chen, 2009). In mid-2008, Re-5 (rg with the HA and NA genes of A/duck/Anhui/1/2006, clade 2.3.4) became the primary vaccine seed strain and was used until early 2012. In mid-2012, Re-6 (rg with the HA and NA genes of A/duck/Guangdong/S1322/2010, a 2.3.2 clade virus from China) became the primary vaccine seed strain. In addition, two specialty rg vaccine seed strains were developed for targeted use: 1) Re-4 (HA and NA from A/chicken/Shanxi/2/2006 a clade 7 isolate) used only in Shanxi and Ningxia provinces in China during 2006–2007, and Re-Egy (HA and NA from A/chicken/Egypt/18-H/2008 a clade 2.2.1 isolate) used in Egypt beginning in 2011 (Chen, 2009). All of these rg vaccines used the PR8 backbone which replicates well in eggs and were produced by the group at Harbin Veterinary Research Institute.

By far the majority of H5N1 vaccine used has been inactivated oil-emulsion adjuvanted vaccine. Vectored vaccines have been used, although more sporadically since they are licensed less widely. China developed and used their own fowl poxvirus (FPV) vectored and Newcastle disease virus (NDV) vectored vaccines for chickens which expressed the HA and NA from A/goose/Guandong/1996 (Ge et al., 2007; Qiao et al., 2009, 2006). Herpesvirus of turkeys (HVT) vectored vaccines with an AIV H5 gene insert have been developed and commercially produced, and they have been shown to be efficacious in experimental conditions (Rauw et al., 2011), but HVT vectored vaccines have only been licensed for use in Egypt and the USA (i.e. for emergency use in the USA) and no field data has been reported.

### 1.4. Application strategies in the field

How vaccine for H5N1 in chickens is applied and the details of vaccination programs vary widely because of the differences in situations with each region, industry compartment and challenge in the field. There are several functional approaches to vaccination: preventive, emergency, and routine or systemic all of which have been reviewed thoroughly elsewhere (Marangon et al., 2007). Briefly, preventive vaccination is used where there is no current infection, but the risk of introduction is high. During preventive vaccination, high risk populations are vaccinated for the duration of the threat. Emergency vaccination is a short term program which targets unaffected animals which are in close proximity to an outbreak and lasts until the outbreak is controlled. Emergency vaccination is often applied as ring vaccination where only animals in a geographic risk zone are vaccinated and may include vaccination within the

**Table 1**

Isolates used as seed strains for inactivated, oil-emulsion vaccines for immunization of chickens against H5N1 HPAIV.

Isolate	Lineage	Notes	Use
A/chicken/Hidalgo(Mexico)/1994 H5N2	Classical		Widespread
A/turkey/England/N28/1973 H5N3	Classical		Limited
A/turkey/Wisconsin/68 H5N9	Classical		Limited
A/chicken/Italy/22A/1998 H5N9	Classical		Limited
A/chicken/Legok(Indonesia)/2003 H5N1	Clade 2.1.1		Limited
A/duck/Novosibirsk/02/2005 H5N1	Clade 2.2		Limited
A/goose/Guandong/1996 H5N1	Clade 0	Re-1, rg <sup>a</sup> in PR/8 backbone with LP PCS <sup>b</sup>	Widespread
A/chicken/Shanxi/2/2006 H5N1	Clade 7	Re-4, rg in PR/8 backbone with LP PCS	Limited in China
A/duck/Anhui/1/2006 H5N1	Clade 2.3.4	Re-5, rg in PR/8 backbone with LP PCS	Widespread
A/duck/Guangdong/S1322/2010 H5N1	Clade 2.3.2	Re-6, rg in PR/8 backbone with LP PCS	Recently introduced
A/chicken/Egypt/18-H/2008 H5N1	Clade 2.2.1	Re-Egy, rg in PR/8 backbone with LP PCS	Limited in Egypt

<sup>a</sup> rg = reverse genetics generated reassortant.

<sup>b</sup> LP PCS = low pathogenicity proteolytic cleavage site in HA.

outbreak zone. The scope of the risk zone will vary depending on the specifics of the outbreak. Finally, routine vaccination is a long term program which is implemented when the virus has become enzootic.

Specific details of how routine vaccination for H5N1 in poultry has been administered depends on numerous factors such as whether it is government or industry driven (or both), biosecurity, the types of *at-risk* populations (village poultry, layers, meat birds, breeders, etc.) and available resources. Industry driven programs in developed countries have focused more on valuable long-lived birds, such as layers and breeders than meat-type poultry. However, because of the high risk of poultry infections in the four H5N1 enzootic countries, meat-type poultry have received the majority of vaccine, and chickens have been the dominant species, which reflects the numeric production of poultry species in agriculture of the individual countries (Swayne et al., 2011). In many developing countries, the sector 4 or village poultry comprise over 50% of the poultry production, but the coverage rate in village poultry is insufficient to achieve population immunity with reports of low coverage in different districts of Egypt (0–40%) and Indonesia (<20%) (Egypt, 2010; Mariner, 2011). Coverage rates are reported to be higher in commercial poultry in Egypt (50–60%) (Peyre et al., 2009b). Government vaccination programs in Viet Nam have had a higher coverage rate in especially ducks, but participation is falling (Swayne et al., 2011).

Hong Kong has been able to accomplish a uniquely high level coverage (near 100%) in meat chickens because of their unique industry structure where 80% of chickens are imported from China through a single government-controlled wholesale market and distributed to 30 clusters of retail live poultry markets. Only 20% of the meat broilers are grown in Hong Kong in small sector 3 farms under production permits with government oversight. These chickens are hatched and imported at day of age from China; thus there is a lack of long lived breeders in Hong Kong (Swayne et al., 2011). Also, there is no village production of poultry in Hong Kong. In conjunction with the preventive vaccination program, the unique industry structure; improved biosecurity controls for importation, production and marketing, and the small size of live poultry market system have resulted in successful control of H5N1 HPAI virus in Hong Kong despite periodic introductions from southern China.

On the contrary, in Egypt, for example, the targeting of meat birds (chickens and turkeys) with vaccination has been due to the high risk of infection (routine vaccination program). Egypt has a more typical integrated commercial poultry industry as well as 40–60% of production in small holder operations. Due to the combination of poor biosecurity and high density of operations, vaccinating meat birds has been seen as a necessity and economically viable (Grund et al., 2011; Kilany et al., 2011).

National flock immunity has only been achieved in Hong Kong. Other countries have vaccine coverage below minimum threshold

for herd immunity, which is 60–80% (Swayne et al., 2011). This has hampered effective vaccination programs and prolonged the H5N1 panzootic. Countries where the virus is not enzootic have used vaccination programs that target high risk regions or poultry types, and not nationwide campaigns. This targeted approach has assisted in eradication effects, in conjunction with stamping-out programs.

## 2. Experimental studies with current and new vaccine technology

Despite the relatively limited use (geographically) of vaccine to control H5N1 in poultry, new approaches and platforms for vaccines are continually reported in the literature. Importantly much of the emerging vaccine technology for H5N1 vaccine has been developed for potential public health use, not necessarily veterinary use because the H5N1 HPAIV and other AIVs are seen as potential pandemic viruses. Therefore the H5N1 HPAIV lineage has become a proof-of-concept target for research and development of vaccine technology for application in humans. For this reason there are numerous reports of challenge studies for novel H5N1 vaccine platforms performed in mice, ferrets and even macaques. A few studies have evaluated novel vaccine technology for H5N1 in chickens and will be described in this review. Chickens represent the vast majority of gallinaceous poultry and minimal experimental vaccine work for H5N1 HPAIV specifically in turkeys has been reported (Kilany et al., 2011).

### 2.1. What we know about current vaccines

There are still some critical gaps in our knowledge of vaccines for AIV in chickens. Much of the experimental work with chickens has been to evaluate vectored vaccines. Considering that most of the vaccines used in the field are inactivated oil-emulsion vaccines, there is relatively little experimental data for improving these vaccines in the literature; most relates to refining these vaccines by generation of vaccine seed strains by reverse genetics and studies to evaluate different seed strains with different field viruses (i.e. evaluations of antigenic matching).

It should also be noted that experimental studies cannot completely simulate field conditions where the birds can be immunosuppressed from other infectious agents and/or environmental stress. Experimental conditions also frequently use lighter lines of chickens and may not reflect the variations among chicken genetic lines in immune response and physiology. Although the specifics for AIV have not been reported, breed and genetic lines are known to affect susceptibility to disease and infection for numerous diseases in chickens (Hassan et al., 2004; Nielsen et al., 1998; Smith et al., 1985). Therefore vaccine efficacy reported in the

literature from lab experiments will frequently be higher than what may be experienced in the field.

The most reliable data for the efficacy of a given vaccine with a target AIV isolate is *in vivo* studies in the target species. Ideally one could extrapolate protection based on antibody titers to a target isolate (the challenge virus), however data on the correlation of antibody titer (most frequently determined by hemagglutination inhibition (HI) assay) with protection against mortality, morbidity or shed is inconclusive and even contradictory. Different studies have suggested that HI titers to the challenge virus in the range of 32–40 may be protective against mortality (Liu et al., 2003; Wood et al., 1985), and that titers over 139 can substantially decrease replication (Swayne, 2006). Conversely, numerous reports demonstrate protection from mortality with HI titers below 40, including some birds that lacked detectable HI antibodies (Abbas et al., 2011; Kumar et al., 2007; Swayne et al., 2001; van der Goot et al., 2005). This may indicate that titers above a certain threshold are predictive of protection, but are not predictive below this threshold. Establishing a relationship between antibody titers and protection may also be hindered by lack of harmonization in the procedures for evaluating vaccine efficacy in chickens. There are no standards for vaccine dose, time between vaccination and challenge, challenge virus dose, or route of challenge virus inoculation, all of which can affect observed protection.

### 2.1.1. Duration of immunity

A critical factor in vaccination is the duration of immunity. However, because studies to evaluate duration of immunity are logistically difficult and expensive to conduct, relatively few have been reported. For those that have been reported results have varied widely and are difficult to compare because of differences in experimental design. For example, one study showed that chickens were protected against mortality from homologous clade 1 challenge with 0.5 ml of  $10^5$  EID<sub>50</sub>/ml for 12 weeks when a specific dose of antigen (at least 1.25 µg/dose) in an inactivated vaccine prepared with a commercial oil adjuvant was used (Hwang et al., 2011). This same study also demonstrated that 4 times as much antigen was needed to protect against an H5N1 virus of a different clade (clade 2.3.4) versus homologous challenge (Hwang et al., 2011). One study evaluated a vaccine prepared with mineral oil and anhydromannitol-octadecenoate-ether adjuvant in five chickens and showed protection to H5N1 HPAIV challenge with  $10^2$  50% chicken lethal doses (no mortality or morbidity) 138 weeks after vaccination (Sasaki et al., 2009). Another study looked at the persistence of antibody in chickens and ducks in the field to determine the duration of immunity from an inactivated oil-emulsion adjuvanted commercial clade 1 H5 vaccine (Boltz et al., 2009). They found that antibody to the vaccine virus could only be detected by hemagglutination inhibition (HI) assay 1 month post vaccination in 20% of the birds, but when 2 doses of vaccine were administered 8 weeks apart, antibody was detectable for up to 10 months post vaccination, although the titer did decline substantially after about 5 months (Boltz et al., 2009).

### 2.1.2. Effect of vaccine on infectious dose

The most critical characteristic of vaccination in stopping virus transmission is its effect on the infectious dose (quantity of virus at exposure) needed to infect an animal. Minimum infectious dose (MID) studies are more cumbersome and resource intensive than typical vaccination-challenge studies, but can offer some valuable information. At this time only a very few have been reported for AIV vaccines in poultry and the results vary based on conditions. Capua et al. demonstrated a  $10^2$  higher dose of a HP H7N1 isolate was required to infect turkeys vaccinated with a commercial inactivated vaccine versus non-vaccinated turkeys (Capua et al., 2004). Bublot et al. demonstrated that a  $10^5$  higher dose of an H5N1 HPAIV

was needed to infect chickens vaccinated with a fowl-pox recombinant vectored vaccine versus non-vaccinated chickens (Bublot et al., 2007). In addition, when vaccinated poultry become infected, they excrete less virus into the environment which results in breaking the infection cycle by reducing the environmental load of the virus (Suarez et al., 2006; Swayne et al., 1997).

## 2.2. Making better H5N1 vaccines for poultry: new technology

Characteristics of the perfect AIV vaccine for poultry have been reviewed and none of the current vaccines meet all of these criteria (potent, safe, stable at ambient temperatures, cheap, and effective after one dose) (Peyre et al., 2009a). Some additional features which the “perfect” AIV vaccine for poultry would have is to be compatible with a reliable and sensitive system for differentiating vaccinated from infected animals (DIVA), and the ability to apply the vaccine without handling each bird individually (mass application). It should also be recognized that some of the problems with vaccination are not going to be solved directly by technical advances. For example there are the political/trade issues because vaccination has been used as a non-tariff trade barrier. Reliable systems for DIVA would be very helpful for maintaining markets if policies could support them. There are numerous DIVA strategies some of which have been used fairly successfully in the field, but since DIVA has been recently reviewed comprehensively elsewhere (Suarez, 2012) we will not discuss DIVA in more depth. Improving AIV vaccines in these areas has been the focus of much recent research, which is described below (Table 2).

### 2.2.1. Antigenic matching

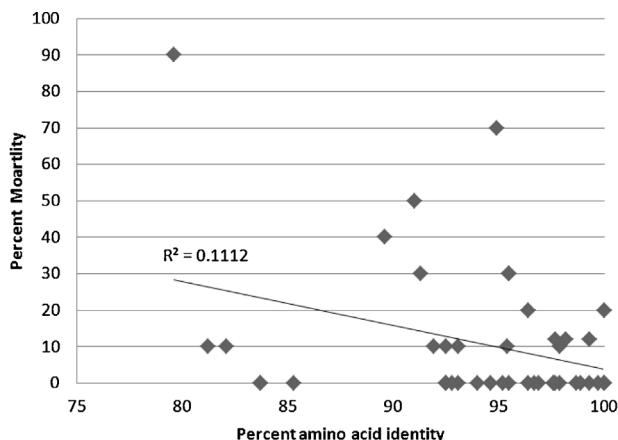
Antigenic matching of the HA in a vaccine to the HA which is in the field virus has been recognized as one of the most critical factors in influenza A vaccine efficacy for all species for a long time. As initially seen with human influenza viruses, AIV has a strong propensity to mutate and antigenic variants generated by mutation are frequently observed within a few years of influenza transmitting to a new population (excluding natural host reservoirs). Antigenic switches or shifts, which are due to reassortment where a new HA subtype entirely is gained, are a different kind of problem for vaccination and are rarer and more easily recognized. Antigenic drift may be more difficult from a practical perspective because currently there are no accurate *in vitro* tests to determine whether a virus has drifted to a point where a given vaccine can no longer provide adequate protection. Although there are some *in vitro* and *in silico* tools, for example hemagglutination inhibition (HI) assay data which can be mapped with antigenic cartography (Cai et al., 2010; Fouchier and Smith, 2010; Smith et al., 2004), and protein sequence data, which provides insight when a vaccine does not protect adequately in the lab or field. As more information is gained on the antigenic importance of specific epitopes and residues the hope is that one day these tools can provide more definitive information.

An interesting characteristic to note is that the few H5 AIV vaccine studies using viruses and vaccines up to the time the H5N1 HPAIV became enzootic in Asia (in late 2003) demonstrated that even isolates with relatively unrelated H5 HA's based on amino acid sequence (e.g. <88% identity) could provide protection against most H5 AIVs including the early H5N1 isolates (Swayne et al., 1997, 2001; Swayne et al., 2006, 2000c) (Fig. 1). Now H5N1 isolates with about 97–98% amino acid identity in the HA may not be cross protective, because the variation is at critical antigenic sites. Importantly, although there is less data on the H5N2 LPAIV in Mexico, available data suggests a similar pattern of variation predominantly at critical antigenic sites (Escorcia et al., 2008; Lee et al., 2004; Villarreal, 2009). Clearly the mutations are being selected for antigenic escape. What this does not tell us is whether the

**Table 2**

**Table 2**  
Novel and experimental technology for poultry vaccines for highly pathogenic avian influenza virus.

Technology	Advantages	Disadvantages
<b>Live vaccines</b>		
<b>Vectors</b>		
Fowl pox virus	<ul style="list-style-type: none"> <li>• Administration with another common vaccine</li> <li>• DIVA compatible since only has AIV HA gene</li> </ul>	<ul style="list-style-type: none"> <li>• Active immunity to FPV will decrease efficacy</li> <li>• Needs cold storage</li> <li>• Requires handling of individual birds for administration by injection</li> <li>• Long term safety unknown</li> <li>• Needs cold storage</li> <li>• Active immunity to NDV will decrease immune response</li> <li>• Maternal antibody to NDV will inhibit immunity</li> <li>• Needs cold storage</li> <li>• Requires handling of individual birds for administration</li> </ul>
Newcastle disease virus	<ul style="list-style-type: none"> <li>• Administration by mass application</li> <li>• Administration with another common vaccine</li> <li>• Induces immunity in the respiratory system</li> <li>• DIVA compatible since only has AIV HA gene</li> </ul>	
Herpesvirus of turkeys	<ul style="list-style-type: none"> <li>• Administration by mass application (<i>in ovo</i>)</li> <li>• Administration with another common vaccine</li> <li>• Minimizes interference from maternal antibodies (with <i>in ovo</i> administration)</li> <li>• DIVA compatible since only has AIV HA gene</li> <li>• Induces mucosal immunity</li> <li>• Administration by mass application</li> <li>• DIVA compatible since only has AIV HA gene</li> </ul>	
<i>Salmonella spp.</i>	<ul style="list-style-type: none"> <li>• Induces immunity in the respiratory system</li> </ul>	<ul style="list-style-type: none"> <li>• Long term safety unknown</li> <li>• Unproven, challenge data for chickens shows incomplete protection with current constructs</li> </ul>
<b>Live attenuated vaccines</b>		
Cold adapted, replication impaired		
<b>Inactivated vaccines</b>		
Whole virus (currently in wide use)	<ul style="list-style-type: none"> <li>• Safe</li> <li>• Efficacious with adjuvants</li> </ul>	<ul style="list-style-type: none"> <li>• Requires handling of individual birds for administration</li> <li>• Most effective with multiple doses</li> <li>• Changing antigen may be cumbersome</li> <li>• DIVA more difficult</li> <li>• Requires handling of individual birds for administration</li> <li>• Cost prohibitive with current technology for production</li> <li>• Requires multiple doses</li> <li>• Requires handling of individual birds for administration</li> <li>• Cost prohibitive with current technology for production</li> <li>• Requires multiple doses</li> </ul>
DNA	<ul style="list-style-type: none"> <li>• Antigen easily updated</li> <li>• DIVA compatible since only has AIV HA gene</li> </ul>	
<i>In vitro</i> produced antigen (plant or <i>baculovirus</i> expressed)	<ul style="list-style-type: none"> <li>• Antigen easily updated</li> <li>• DIVA compatible since only has AIV HA gene</li> </ul>	
<b>Adjuvants</b>		
Mineral oil	<ul style="list-style-type: none"> <li>• Inexpensive</li> <li>• Efficacious</li> <li>• Induces good immunity</li> </ul>	<ul style="list-style-type: none"> <li>• Requires handling of individual birds for administration</li> <li>• Can only be used with vaccine platforms (e.g. DNA) which are cost prohibitive.</li> <li>• Unproven, no challenge data for AIV available for chickens, turkeys</li> </ul>
CD154		
Chitosan, liposomes	<ul style="list-style-type: none"> <li>• Effective mucosal adjuvant</li> </ul>	



**Fig. 1.** Protection from mortality by amino acid identity between vaccine seed strain and challenge virus for specific pathogen free chickens vaccinated once with an inactivated oil emulsion vaccine and challenged 3 weeks later (Swayne et al. Veterinary Microbiology 2000; Swayne et al. Avian Pathology 1999; Spackman, unpublished data).

diversification of H5 AIV antigenically is due simply to the persistence of the virus in poultry which have different epidemiological dynamics than natural hosts, or due to vaccination.

Currently there are numerous theories about whether antigenic drift is *caused* by vaccination as antigenic drift has been noted in all species for which vaccines are available and used: equine, swine, human and chickens (Abdel-Moneim et al., 2011; Cattoli et al., 2011; de Jong et al., 2007; Escorcia et al., 2008; Lee et al., 2004; Park et al., 2009a; Smith et al., 2004). The theory being that immunity from vaccination selects for antigenic variants, which then become the dominant population. Certainly immunity causes antigenic drift, but the critical question is whether *vaccinal* immunity plays a significant role. Some have even suggested that this is a reason not to vaccinate. Arguably the evidence that vaccinating causes more problems by causing antigenic drift is not conclusive.

First, vaccine coverage within any of these populations (human, swine, horse, chicken) is far from complete, for example, most of the world's human population is not vaccinated for H3 influenza, but steady antigenic drift has been documented since the 1960's (Smith et al., 2004); i.e. antigenic drift with human H3 influenza viruses must primarily be due to immunity from natural infections. Second, vaccination increases the infective dose needed to

propagate infection and even if the vaccinated individual becomes infected they tend to shed lower titers of virus thus limiting their ability to infect the next host. In the chicken model specifically, the amount of virus shed by vaccinated individuals is reduced and is expected to be below the level needed to infect another individual, especially another vaccinated individual. Although there is limited data on minimum infectious dose for many HPAIV strains so this is conjecture, a few reports suggest that most HPAI viruses have mean infective doses for chickens of  $10^3$ – $50\%$  egg infectious doses (range of  $10^2$ – $10^4.5$ ) (Kwon and Swayne, 2010; Swayne and Slemons, 2008). Transmission from vaccinated individuals to other vaccinated individuals is likely limited due to decreased shed and increased resistance to infection.

On the contrary, incomplete immunity from vaccination could provide a good system for virus selection. Transmission from a vaccinated to a non-vaccinated individual (since 100% coverage is not likely) or poor responder to vaccine who could amplify variants is more likely. Using poultry as the example, incomplete immunity to vaccines for many diseases has been documented in chickens for numerous reasons: poor host response due to immunosuppression, inadequate dose either due to vaccine spoilage or intentional cutting of doses to vaccinate more birds with less vaccine, or challenge/exposure to the virus before full immunity to the vaccine has been developed by the host and finally, even a poor antigenic match between the vaccine and field strains of the virus, and, on a population basis, low vaccination coverage rate in the field (Abdelwhab et al., 2011; Chen, 2009; Eggert et al., 2010; Grund et al., 2011; Swayne et al., 2011). Of course poor immunity can arise from exposure to virus naturally for the same reasons, but the bird would typically die, although possibly not before shedding antigenically selected virus. In addition, waning maternal antibody would cause a similar situation where the animal has limited, poor immunity, but could still be infected and could shed virus within a poorly immune population. Therefore the question becomes: *does vaccination on a broad scale induce enough immunity to select antigenic variants of the virus, but not enough immunity to prevent infection or shed?* This probably could happen, but does it happen enough to have a significant impact versus other conditions that would allow for selection of antigenic variants?

This is relevant for vaccination of chickens for H5N1 HPAIV, since chickens, by dose, receive the most vaccine for H5N1 and because antigenic drift has proved to be a crucial problem for H5N1 vaccination as some of the original vaccines, e.g. Re-1 and vaccines based on Mex/94 have reduced efficacy to numerous recent sub-clades of the H5N1 HPAIV or recent variants of the Mexican H5N2 LPAIV (Escoria et al., 2008; Grund et al., 2011; Lee et al., 2004).

Just as importantly, this situation is in no way unique to influenza A; antigenic variation has been a recognized characteristic of numerous other viral diseases of poultry for a long time. Probably one of the best described is infectious bronchitis virus (IBV) (Hofstad, 1958; Lashgari and Newman, 1984; Tevethia and Cunningham, 1968). Although the situation is not exactly the same, the approach to vaccination for IBV, which is routine in chickens in the US, is similar to that of AIV in enzootic areas, the serotypes that are most common in the field are included in the vaccines (which is a live virus vaccine).

**2.2.1.1. Identifying antigenic matches.** Regardless of the specific cause, antigenic drift complicates vaccination for AIV, a problem people have attempted to solve several different ways. The first and most common is to produce a vaccine with a seed strain that is closely related to the viruses in the field at the time of production. This can be accomplished several ways. What has been used with human vaccine production, but somewhat surprisingly not with veterinary vaccines, including swine and equine influenza vaccines, is to frequently update the vaccine seed strain. Each year an

expert group works through the WHO to attempt to predict which antigenic variant will predominate during the upcoming influenza season and will use that strain to produce the vaccine. Of course the prediction is not always accurate and vaccine efficacy rates can vary (Osterholm et al., 2012). For veterinary vaccines a similar approach may be beneficial where autogenous vaccines could be used, but regulatory barriers have made this difficult, although local HPAIV vaccine strains for the H5N1 HPAIVs have been used in Russia and Indonesia, and rg vaccines with updated seeds have been generated in China (Bouma et al., 2008; Chen, 2009; Swayne et al., 2011).

For AIV, identifying a seed strain with sufficient antigenic match to the challenge virus is best accomplished through vaccination-challenge studies (either using one of the few commercial H5 vaccine strains or a current field strain) (Swayne and Kapczynski, 2008b). Initial selection of potential seed strains to test *in vivo* has been done for a long time by using HI or virus neutralization (VN) assay data to look at antigenic relatedness among isolates (Archetti and Horsfall, 1950). However this method can be cumbersome to interpret with more than a few strains and requires mono-specific sera to the target strains, which takes time and resources to prepare. The relatively recently described method of antigenic cartography does aid interpretation by providing a visual map of the HI or VN data (Cai et al., 2010; Lapedes and Farber, 2001; Smith et al., 2004).

Another method, which is probably the fastest, is to use the amino acid sequence of the HA1 region of the HA gene to identify the strains which are most closely related to the challenge virus. Comparing amino acid identity does have limited predictive value as not all amino acid changes are in important antigenic sites; vaccines with identities below 88% with the challenge virus have provided protection with both H5 (Swayne et al., 2000c) and H7 (Abbas et al., 2011) HPAIVs. Overall amino acid identity between the HA1 of the vaccine and challenge virus is not very accurate for predicting protection against mortality (Fig. 1).

Antigenic cartography is expected to be more accurate since it uses serological data, such as HI assay, to determine the relationships among isolates (Cai et al., 2010; Smith et al., 2004). At this time only one functional study has been reported in chickens (Abbas et al., 2011). Abbas et al. evaluated H7 HPAIV and did not see a strong correlation between antigenic proximity and protection, but that study used antigenic cartography retrospectively; the study was not designed to test antigenic cartography as a method to identify potential vaccine strains, but to look at vaccines that were used or which were already identified to be potential seed strains (Abbas et al., 2011).

**2.2.1.2. Eliciting a cross-reactive immune response.** Another approach to immunizing for influenza with its substantial antigenic variation is to produce vaccines which induce an immune response which targets epitopes on the virus that are conserved within and even among subtypes. Among several candidate epitopes, probably the most frequently targeted for broadly protective vaccines is the external domain of the M2 protein (M2e) because it is highly conserved and antibodies to it can inhibit virus replication (Neirynck et al., 1999). However current vaccines do not elicit a sufficient response to M2e, so subunit vaccines with various adjuvants have been identified as a method to induce antibodies to M2e. In chickens, attenuated recombinant *Salmonella enteritidis* (live, attenuated *Salmonella* vaccines are already licensed for use in chickens in the US) that expresses the M2e conserved epitope and the chicken CD154 as an adjuvant, have been evaluated for use as a vaccine (Layton et al., 2009). The construct did induce antibody and protected against morbidity to an H7N2 LPAIV, but did not provide any protection to H5N1 HPAIV challenge indicating that the system has potential, but some modifications need to be made first.

Further work with M2e based vaccines using numerous platforms has shown promise for improving cross protection among variant antigens using a mouse model system. Some examples are where a synthetic M2e peptide was able to protect mice against a 10 LD<sub>50</sub> challenge with both a clade 1 and a clade 2.3.4 H5N1 HPAIV after 3 vaccinations in Freund's incomplete adjuvant (Zhao et al., 2010). Also, Park et al. included M2e epitopes in an adenovirus vectored recombinant HA vaccine to improve cross-reactivity (Park et al., 2009b). M2e vaccines have also been delivered as DNA vaccines, but have been limited in efficacy alone or in combination with other viral genes (Rao et al., 2010). None of the current M2e vaccine formats are economically feasible for poultry due to the high cost per dose, inappropriate viral vectors, and necessity for multiple injections. There is also insufficient data that it can protect chickens against HPAIV challenge.

Another potential "universal" neutralizing epitope that has been described is in the HA stalk region (HA2 region) (Corti et al., 2011; Ekiert et al., 2011). Work with this epitope is still very preliminary and most work has involved monoclonal antibodies, but this is another potential target for inducing more broadly reactive immunity to AIV.

An entirely different approach has been reported by Rao et al., who used DNA vaccines encoding the HA genes of up to 10 different viruses where chickens in some groups received genes from multiple strains (Rao et al., 2008). The results were complex as different injection methods, number of vaccinations and doses were also evaluated, but some groups had some protection against challenge with an isolate of a different clade (Rao et al., 2008). Presently there is no clear new technology for a vaccine to induce cross-clade protection to H5N1 in chickens.

## 2.2.2. Vaccine application

Effective administration of AIV vaccines to poultry is a major concern for influenza vaccination because inactivated vaccines are so labor intensive to use. Vectored vaccines and live-attenuated vaccines have been explored and in the case of vectored vaccines, used in the field as alternatives which can improve application effectiveness through mass application and/or by combining AIV with other vaccinations the birds would receive anyway. A disadvantage to both live-vectored and live-attenuated vaccines is that they are labile to heat, so transport and storage must be able to maintain adequately low temperatures to maintain viable virus.

**2.2.2.1. Vectored vaccines.** Numerous recombinant virus vectored vaccines for H5N1 HPAIV have been reported in the literature; fowl pox virus (rFPV), herpesvirus of turkeys (rHVT), Newcastle disease virus (rNDV), infectious laryngotracheitis virus (rILTV), and avian leucosis virus (rALV) (Swayne and Kapczynski, 2008a). A *Salmonella* vectored vaccine has also been reported (Liljeblad et al., 2010). However, only three vectored vaccines (rFPV, rNDV and rHVT) have been licensed for field use in one or more countries. In addition, various vector systems have been developed that express the influenza HA protein *in vitro* in cell culture systems such as Vaccinia virus, baculovirus, defective adenovirus and defective Venezuelan equine encephalitis virus. None of these are licensed for poultry use and would require injection of individual birds as is necessary with inactivated AI vaccines. Their viability would only be possible if antigen could be produced at a lower cost per unit than whole AIV inactivated vaccines produced in embryonating chicken eggs.

Vectored vaccines offer several advantages over inactivated vaccines: good immune response to a replicating virus (no adjuvant needed, less starting antigen needed), some vectors may be mass applied and both costs and efficiency are improved by giving two vaccinations with the labor required for one. A specific advantage of rHVT vectored vaccines is that they can be given *in ovo* which saves labor and reduces interference from

maternal antibodies, although the duration of immunity is not clear. Potential disadvantages, which has been observed with rFPV vaccines is that they may not confer adequate immunity if the bird has been exposed to or vaccinated with the vector previously (Swayne et al., 2000a). Concerns about the safety of herpesvirus vectored vaccines have been raised through the discovery of novel recombinant virulent ILTV strains with genes from vaccines in the field (Lee et al., 2012). However, since HVT is not virulent, unlike ILTV, this is less of a concern with HVT vectored vaccines.

With the current technology, rNDV vectored vaccines possibly offer the best solution as they can be mass applied through spray in the hatchery or drinking water. Some of the other advantages of NDV are that vaccination of chickens for NDV is routine world-wide, and because the virus is live and replicates in the tissues and organs where AIV does it confers better immunity with less initial antigen than with inactivated vaccines administered parenterally. NDV vectored vaccines using the LaSota strain are being used in China (Chen, 2009; Ge et al., 2007) for H5N1 HPAIV and have been used in Mexico for the H5N2 LPAIV (Villarreal, 2009). A further advantage of NDV vectored vaccines is that NDV will replicate in both chickens and turkeys. On the contrary maternal antibodies to the vector will interfere with development of active immunity, creating limitation on their mass usage or replacement of inactivated vaccines.

Fowl poxvirus-vectored vaccines for AIV were first described in the late 1980's (Taylor et al., 1988) and have been well described in the literature for their efficacy in chickens, both H7 (Boyle et al., 2000; Qiao et al., 2003) and H5 vaccines (Beard et al., 1992; Qiao et al., 2009, 2003; Swayne et al., 2000a, 2000b, 2000c; Webster et al., 1996) have been tested, and different inoculation routes (Beard et al., 1992) and immunization schemes have also been described (Chen et al., 2011; Steensels et al., 2009; Swayne et al., 2000a). Overall FPV vectored vaccines, when used properly, can be efficacious. FPV vaccines have been licensed in the US (although not used), but have been used for H5N1 HPAIV in China and have been used since 1995 in Mexico against H5N2 HPAIV and LPAIV. These vaccines work best as priming vaccine at 1 day of age followed by an inactivated influenza vaccine at 10–21 days later (Steensels et al., 2009).

Another respiratory virus of chickens, ILTV, a herpesvirus, has been experimentally tested as a vaccine vector for AIV vaccines (Luschow et al., 2001; Pavlova et al., 2009a, 2009b; Veits et al., 2003). Although the host range of ILTV is restricted to chickens, the vaccine can be administered by eye drop (mass applicable), replicates in the respiratory mucosa therefore confers immunity in the tissues where AIV replicates, and like other vectored vaccines that utilize only the AIV HA insert, it is theoretically compatible with DIVA strategies which target the internal protein genes of AIV that are lacking in recombinant vaccines. Pavlova et al. showed that a clade 1 H5N1 HPAIV in an attenuated ILTV vector could protect chickens after a single vaccination from morbidity and mortality, as well as reduce shed not only to homologous challenge but to challenge with a clade 2.2 virus (96.1% amino acid identity) and a classical lineage H5 HPAIV (93.6% amino acid identity) (Pavlova et al., 2009b). Presently, no ILTV vectored vaccines have been licensed.

The use of adenovirus vectored vaccines in chickens has also been reported by several groups. Pose et al. used an adenovirus vector to produce a subunit vaccine, although they did not conduct challenge studies, they did evaluate the chicken's immune response to the subunit vaccine which was produced with a mineral oil adjuvant and which had the chicken CD154 molecule as an additional adjuvant (Pose et al., 2011). Another group demonstrated 100% protection from mortality to homologous challenge with 10<sup>6</sup> 50% embryo lethal doses of A/VietNam/1203/2004 after

a single subcutaneous vaccination of chickens with an adenovirus vectored vaccine (Gao et al., 2006).

Some of the more practical work for poultry with adenovirus vectors has been conducted by Toro et al. (practical because of their use of mass application using *in ovo* inoculation) (Toro et al., 2007, 2008). Toro et al. produced an adenovirus vectored vaccine with the insert from A/turkey/WI/1968 (H5N9) classical lineage H5 HA and evaluated challenge of *in ovo* vaccinated chickens with  $10^5$  50% embryo infectious doses of A/swan/Mongolia/244/2005 H5N1 (clade 2.2) (Toro et al., 2007). There was only 68% survival, however they saw 100% survival with a similar study using A/chicken/Queretaro/14588-19/1995 H5N2 challenge, which has 94% amino acid identity to the vaccine versus 89% with the Mongolian virus, indicating that a more closely related vaccine would provide better protection (Toro et al., 2007). Further work by the same group demonstrated that the antibody response to *in ovo* administered adenovirus vectored vaccines could be increased with a higher dose and by using a codon optimized vaccine construct (Toro et al., 2008).

A *Salmonella* vectored vaccine containing an H5 HA insert from A/whooper swan/Mongolia/3/2005 (clade 2.2) was evaluated in chickens against both high and low dose homologous challenge and also against challenge with a classical lineage H5 (A/chicken/Queretaro/14855-19/1995) (Liljebelke et al., 2010). After two weekly inoculations by crop gavage the chickens were protected against mortality from the low dose homologous challenge ( $10^4$ EID<sub>50</sub> per bird), but there was 60 and 100% mortality in each vaccine group with a higher dose challenge of  $10^6$ EID<sub>50</sub> per bird (Liljebelke et al., 2010). Similar dose dependent results were observed with challenge with the classical lineage virus after four weekly inoculations. The *Salmonella* based vaccine also did not appreciably reduce shed in any challenge group (Liljebelke et al., 2010).

**2.2.2. Live attenuated vaccines.** Live attenuated vaccines (LAV) for seasonal influenza have been utilized for some time. Despite the fact that regulatory barriers are probably insurmountable for AIV based LAV in poultry due to the possibility of reassortment with field strains, some experimental work has been reported. Potential advantages are mass application, and potentially better immunity because of replication in the relevant tissues and induction of cellular immunity to influenza. Nonetheless there has been some experimental work with LAV for AIV which has shown that they can provide protection. Several reports have been published on LAV to protect against LPAIV, however since the scope of this review is to focus on H5N1 HPAIV we will include only on selected works on LAV.

Steel et al. developed a LAV recombinant AIV, which also included the NDV HN gene (Steel et al., 2008). Protection to both H5N1 HPAIV and NDV challenge was evaluated in chickens 3 weeks after vaccination at either 2 weeks of age, or *in ovo* at 18 days of incubation. Somewhat interestingly the *in ovo* vaccinated birds were protected against mortality (90% and 80% survival from NDV and HPAIV challenge respectively) and shed reduced levels of virus. In contrast, vaccination at 2 weeks of age provided no protection (Steel et al., 2008).

Cold adapted attenuated influenza vaccines for human immunization were developed many years ago (Davenport et al., 1977). Nang et al. used reverse genetics to produce a cold adapted H5N1 clade 1 A/VietNam/1203/2004 virus with the HA proteolytic cleavage site mutated to a LP sequence in a backbone of an H9N2 LPAIV and demonstrated protection to mortality with homologous challenge which was improved after two vaccinations versus one (Nang et al., 2012).

Another live H5N1 vaccine used the same clade 1 strain (A/VietNam/1203/04) also with the HA engineered to be LP, but

introduced mutations into the PB2 and truncated the NS1 protein to attenuate the virus (Steel et al., 2009). Chickens were vaccinated once and challenged with the homologous HPAIV strain or a clade 2.2 virus and were protected from mortality (100% survival in the homologous challenge group and 84% in the heterologous challenge group) and virus shed was below detectable limits for both challenge groups (Steel et al., 2009).

### 2.2.3. Vaccine production

Some of the technology reported recently for veterinary use includes methods (frequently using reverse genetics to add the appropriate HA and/or NA to a virus which already grows well in culture) to produce antigen for killed vaccine more efficiently as it is difficult to achieve an adequate antigenic load with the H5N1 HPAIV lineage because it kills the chicken embryo or cultured cells too quickly if field virus is used. Improving replication in embryonating chicken eggs (ECE) has been a common focus (Abt et al., 2011; Harvey et al., 2010; Isoda et al., 2011; Zhang et al., 2011). However, it would be advantageous to eliminate the need to grow virus in ECE as they are expensive and have a complicated supply chain therefore numerous groups have reported growing virus with high efficiency in cell culture, often VERO or MDCK cells (Coussens et al., 2011; Tambyah et al., 2012; Tseng et al., 2011; Zhang et al., 2011; Zhou et al., 2012b).

In addition to the use of reverse genetics to engineer viruses with the antigenically relevant HA and NA which replicate well in culture, and where the H5 HA has been modified to contain a LP cleavage (Chen, 2009) as there are concerns with using an HP virus in a vaccine because of the biosecurity needed to grow an HPAIV to high titers and in large quantities.

Another strategy has been to develop alternative platforms for producing antigen for non-infectious vaccines. Two of the most common are DNA vaccines and protein expression to produce sub-unit vaccines in other systems like plants or in cell culture systems. The latter has used various systems, like baculovirus, with the inserted influenza HA gene.

The majority of DNA vaccine work has been conducted in mice and is intended to inform pandemic vaccine development, rather than poultry vaccines (Epstein et al., 2002; Kodihalli et al., 1999; Patel et al., 2012; Tao et al., 2009; Tompkins et al., 2007; Xu et al., 2011; Zheng et al., 2009; Zhou et al., 2012a). The cost of DNA vaccines and the fact that they must be administered to animals individually precludes their use in poultry without substantial technological improvements. If those barriers could be overcome a few groups have demonstrated that DNA vaccines will work in chickens although multiple doses are generally needed (Jiang et al., 2010; Rao et al., 2008; Suarez and Schultz-Cherry, 2000). Rao et al. demonstrated protection (no morbidity, no mortality, no detectable virus shed) of chickens to H5N1 HPAIV challenge after two doses of DNA vaccine using a challenge dose of 20 50% lethal doses (Rao et al., 2008). Similar results have been seen with a DNA vaccines for H7 HPAIV (Jiang et al., 2010) as well. A third study using a baculovirus vesicular stomatitis virus pseudotyped vector only showed complete protection of chickens with two inoculations of the highest dose against a  $10^2$  50% lethal dose challenge with H5N1 HPAIV (Wu et al., 2009). For comparison most inactivated influenza vaccine challenge studies utilized a minimum of  $10^4$  50% lethal doses or  $10^6$  EID<sub>50</sub> for the challenge (Abbas et al., 2011; Eggert and Swayne, 2010; Grund et al., 2011; Kilany et al., 2011; Swayne et al., 2001, 2006, 2000c).

Subunit vaccines to H5 using HA protein produced to high quantities *in vitro* with a baculovirus expression system have been evaluated in chickens. Mortality, morbidity and shed were reduced after a single inoculation with vaccine using A/Hong Kong/156/1997 H5N1 HPAIV homologous challenge (Swayne et al., 2001). Protection was markedly better with the higher of the 2

doses evaluated. Lin et al. also evaluated different doses in mineral oil adjuvants (Lin et al., 2008) and observed a dose effect where higher quantities of antigen induced higher antibodies levels and there was a correlation between high antibody titer and both reduction in clinical disease and virus shedding (Lin et al., 2008).

A few studies using other methods such as plant based production of antigen have been reported (D'Aoust et al., 2010; Landry et al., 2010), but have focused on pandemic vaccines and none have been tested in chickens.

#### 2.2.4. Adjuvant technology

Developing superior adjuvants has been attempted with hopes that better adjuvants will improve antibody titers, the duration of immunity and even the breadth of immunity. The majority of adjuvant research reported in the literature is for pandemic vaccines (human use); no work has been reported recently for inactivated AIV, which have evaluated alternatives to the standard of mineral oil currently used in numerous poultry. Importantly, the current mineral oil adjuvants are cheap and work very well. Also the focus of new technology for poultry vaccines has been to find alternatives to inactivated vaccines which must be applied by hand to individual birds.

Work with adjuvants for vaccines for other poultry diseases probably has little relevance for AIV, unless one of these agents is used as a vector, such as live NDV vectored AIV vaccine which has been used in Mexico and China (Ge et al., 2010; Romer-Oberdorfer et al., 2008; Villarreal, 2009). Work with adjuvants for live NDV vaccines to stimulate mucosal immunity has evaluated chitosan (Rauw et al., 2010) and liposomes (Tseng et al., 2010).

#### 2.2.5. Abrogating interference from maternal antibodies

Maternal antibody interference with vaccination of young poultry has been recognized for a long time and has been a hurdle to vaccinating chicks and pouls at the hatchery for numerous diseases (Box, 1965; Mondal and Naqi, 2001; Naqi et al., 1983). Hatchery vaccination, including *in ovo* administration of vaccines, has numerous advantages, for example saving resources due to logistics as well as immunizing the animals as early as possible. Since meat birds are rarely vaccinated for AIV, this has not been a major problem until recently when poultry producers in Egypt began to vaccinate young chicks that were the progeny of breeder flocks with high antibody levels. Experimental work with progeny from vaccinated breeders and passively immunized 7 day old chicks indicated that maternal antibodies could interfere with vaccination (Abdelwhab et al., 2012; Kim et al., 2010; Maas et al., 2011). Although maternal antibodies have been blamed as a factor in the difficulty in controlling AIV in Egypt (Kim et al., 2010), biosecurity is probably a more important factor; AIV has been controlled in numerous countries where birds are not vaccinated at hatch or within the first 2 weeks of life, if at all.

Currently, no specific strategy for abrogating the interference by maternal antibodies in poultry at hatch has been implemented for H5N1, but vectored vaccines have been utilized for other diseases of poultry and have been reported to be advantages of both HVT and FPV vectored vaccines (Bublot et al., 2006; Rauw et al., 2011).

### 3. Conclusions

Vaccination for H5N1 HPAIV will likely continue for some time as there is no clear indication that the H5N1 HPAIV will be eradicated from poultry in the near future. It is important to recognize that historically vaccination has never been considered an adequate control method for HPAIV on its own. Successful eradication has been accomplished without vaccination in numerous cases (Elbers et al., 2004; Rojas et al., 2002; Senne, 2007) and when vaccine has been used during a successful eradication program other measures

have always been implemented (Ellis et al., 2004; Marangon et al., 2007; Naeem and Siddique, 2006). At the very least appropriate surveillance of both vaccinated and non-vaccinated populations must be maintained. Additionally, there is conjecture that under some circumstances using a poor vaccine or vaccinating inadequately may be worse than not vaccinating at all because infection could be masked. The thought is that mortality and disease would be reduced or even eliminated, but shed would not be eliminated (Garcia et al., 1998), therefore virus would still be excreted into the environment, so the transmission cycle would not be halted.

The contributions of new technology to vaccination and the approach to vaccination are not clear yet. Economics will probably be the most important factor in what new technology is adopted and how it is applied. Also, as the largest user of H5N1 vaccine in chickens by dose (90%) (Swayne et al., 2011) and a country where H5N1 will likely remain enzootic for some time, China will also continue to be the driver of new technology and updated vaccines. Vaccination for H5N1 HPAIV is a complex issue with scientific, practical and political considerations that will continue to evolve with technology and as the field situation continues to change.

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